FINAL REPORT

Technical Proposal No. BB321-55-407P
Contract NAS9-12050

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Lyndon B. Johnson Spacecraft Center
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Analytical and Radio-histo-chemical Experiments of Plants & Tissue Culture Cells Treated with Lunar & Terrestrial Materials

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The objective of the research performed under this contract was to examine more critically the nature and mechanisms of the apparent stimulation of growth originally observed in plants growing in contact with lunar soil during the Apollo Project quarantine. Preliminary experiments employing neutron-activated lunar soil had indicated uptake of a few elements by plants. The originally planned approach was to utilize this technique for further exploration of the problem. Our progress and the evolution of our thinking has been given in detail in the vairous monthly progress reports. In summary, it was found that while the preliminary neutron activation technique allowed demonstration of uptake of minerals it presented numerous disadvantages for use in critical experiments directed at elucidating possible mechanisms of stimulation. Among these were the following major considerations:

1. The extreme difficulty of handling and hazards of possible contamination of plants and experiments by the finely divided mineral material.
2. The very small amounts of measurable radioactivity that were taken up by the test plants if samples were activated for the short periods necessary to give minimal damage to mineral samples and allow measurement of uptake of short half-life isotopes.
3. The fact that the nature of the mineral sample was changed by neutron bombardment thus possibly destroying or seriously altering the properties responsible for growth stimulation.

The approach taken was to use standard activation analysis techniques to study uptake of minerals into plants ofter growth in contact with experimental mineral samples. The experimental portion of this contract therefore consisted

essentially of studies of growth of germ free test plants upon varying amounts of model terrestrial mineral samples and upon lunar soil. The analytical portion of this work will be carried out by The Center for Trace Characterization, TAMU and will be reported separately. The results of other aspects of the work have been given in monthly reports. Our conclusions drawn from this work and from preliminary results of elemental analysis are summarized as follows:

1. There are definite differences in the amounts of various mineral elements taken up by plants growing in lunar soil as compared to other mineral samples.
2. There are also differences in amounts of elements taken up by plants growing in contact with different kinds of terrestrial minerals.
3. These differences in mineral content are probably related to observed differences in growth and coloration.
4. The stimulation noted in the case of lunar soil is not unique and is a difference of degree rather than kind, the governing factor probably being that of availability which may be related to the unusual history of lunar minerals.
5. Growth and general health of plants improved in the presence of relatively small amounts of raw mineral but became inhibited as amounts are increased. Very probably the same pattern would be observed for lunar soil if it were tested in the same way.

The following summarizes the progress of Contract NAS9-12050 which was activated July 1, 1971. Actual work was initiated July 10. Tasks covered by this report are: 1.2.1, a and b of, Exhibit A, Statement of Work for the Investigation of the Effects of Lunar Materials upon Terrestrial Vegetation.

Selection of Varieties for Testing

Twelve varieties are currently being tested; 11 are varieties supplied by Burpees; one by Asgrow. These are listed in Table 1. Other varieties have been ordered, but not received.

Screening for Ease of Decontamination

Decontamination procedure. Weighed seed samples calculated to contain an average of 100 + 5 seeds were placed in sterile, foil-covered 50 ml beakers and surface sterilized according to 2.7.3 C of Botanical Test Protocol MSC 03267 with the following exceptions: (1) A filtered air transfer chamber was employed in place of a laminar flow hood. Bench surfaces were wiped with 5.25% NaOCl diluted 1:5 and the entire chamber was exposed to a UV germicidal lamp for 1 hour prior to use; (2) All seeds that floated on the surface of the sterilizing solution were discarded. Following treatment seeds were planted approximately 50 seeds per plate onto agar plates or 1 seed per tube in screw cap tubes containing sterility test media.

Test media. All agar media were poured 20 ml/plate in sterile 100 x 15 mm styrene plates. Water agar for assay of viability was 1 percent Fisher brand agar in distilled water. Mold and bacterial contamination were tested

respectively in Potato Dextrose agar and Trypticase Soy agar. Broth cultures were 150 x 16 mm screw cap tubes (neoprene lined caps) containing either 5 ml of Trypticase Soy broth or 10 ml of NIH Thioglycollate broth to which had been added agar to 0.75 g/liter to aid in maintenance of anaerobic conditions. All media except agar were Difco brand.

Cultural conditions. Agar media for assay of fungal or microbial contamination were incubated in the dark at 25°C. Liquid media were incubated at 25°C and at 37°C. Initial tests of seed viability have been at 25°C in the dark and with normal day light.

Viability After Sterilization and Storage

Seeds surface sterilized and rinsed as indicated above were placed in sterile petri plates in sterile filter paper. After storage in the dark at 25°C for various periods of time seeds were placed on agar media for assay of viability and contamination. Filter paper lined storage units were prepared by placing a 9 cm. circle of #3 Whatman paper in a glass petri dish, and saturated with 70% ethanol. Units were wrapped in Kraft paper and autoclaved 20 min at 15 lb. Following autoclaving, units were dried in an oven at 120°C for 2 hours.

Results of Testing

Results obtained thus far from tests of viability and contamination testing are given in Table 1. Contamination by aerobic organisms was less than 1 percent in all cases noted. Tests for anaerobic organisms have thus far for yielded negative results. It is our impression (now being tested) that there is some selection for viable and uncontaminated seed if floating seeds are discarded during the decontamination procedure.

Also the procedure outlined in the protocol manual may, in our hands at least, be somewhat severe for certain varieties of seeds as indicated by a high incidence of damaged seedlings. Dark seeded varieties seemed more resistant than light seeded. It is also our impression that there is considerable variation in the nutritional or environmental requirements of different varieties. This will be the subject of extensive study during the following months.

Evaluation of viability of seeds following treatment and storage has been underway for only 1 week. A complete set of initial data will not be presented until the next month. Preliminary results indicate that germination percentage is unaffected, but under the procedures in current use, emerging seedlings from stored seeds show signs of damage in certain varieties.

Discussion

This work is still in an exploratory stage. We have based our initial trials upon procedures outlined in the Botanical Test Protocal Manual. These will serve as a point of departure for development of procedures better suited to our specific needs. The data presented here are meant primarily to be an indication of direction to be taken in further work.

Table 1. Germination rate and contamination of lettuce seeds after surface sterilization by buffered hypochlorite-Triton X-100 solution.

Lettuce Variety	Germination [1] Percent at Days: 2	3	4	6	Percent Seedling Damage	Contamination
Imperial 847 (D) [3]	61	75	85	87	4	Bacterial
Paris Island (L)	44	74	77	87	19	0
Great Lakes (L)	68	90	91	93	21	0
Burpees Iceberg (L)	69	80	92	97	27	0
Dark Green Boston (L)	65	87	95	97	28	0
Salad Bowl (D)	97	98	99	99	0	Mold
Tom Thumb (D)	95	97	97	98	1	0
Grand Rapids (D)	100	100	100	100	0	0
Ford Hook (D)	98	99	100	100	0	Mold
Mesa 659 (L) [2]	7	11	12	17	17	0
Oak Leaf (L)	65	90	97	97	39	0
Butter Crunch (D)	62	87	93	97	30	0

1/ Germination was considered to have occurred when the seed coat had ruptured and a visible root tip had emerged. Percentage is based on counts of 200 seeds.

2/ Supplied by Asgrow. All others supplied by Burpees.

3/ (D) - dark seeded. (L) - light seeded.

The following summarizes the progress of Contract NAS9-12050 for the period August 1 through August 31, 1971. Tasks covered by this report are: (1) 1.2.1 a and b of Exhibit A, Statement of Work for the Investigation of the Effects of Lunar Materials Upon Terrestrial Vegetation and (2) a summary of the work on the selection of another species for testing under 1.2.4 of Exhibit A.

Screening of Lettuce Varieties

Procedures are as described in the monthly report dated August 1. Summarized in Tables 1 and 2 are the results of experiments designed to optimize conditions for surface sterilization of lettuce seeds and to provide further information on the microbial contaminants present in lettuce seeds. There is a great deal of variability between varieties with respect to their resistance to the surface sterilization treatment. The job of selecting the best varieties may become slightly messier than anticipated, but in general it seems that the more resistant varieties are also the fastest growing. We used size classes (Table 2) as an index of damage by treatment. It seems to parallel germination rate fairly well; either will serve as a basis for evaluation in further work. The practice of discarding floating seeds eliminates a high proportion of inviable seeds in some varieties but not contaminated ones. Length of treatment time seems to have little effect upon contaminants, and further, only the varieties indicated in the August report seem to be contaminated. We are initiating tests to ascertain whether this is variety associated or seedlot associated. The fact that contaminants are unaffected by length of sterilization time is discouraging with respect to the goal of development of efficient surface sterilization

procedures, but does provide encouraging evidence that seeds of lettuce may be obtained in a "naturally clean" state. We are attempting to obtain information from the seed companies with regard to the pretreatment undergone by lettuce seeds. Efforts to culture anaerobic organisms (as detailed in the previous report) from seeds apparently free of aerobic organisms have been unsuccessful.

Tests of effect of storage upon viability were re-initiated after the effect of sterilization time upon viability was recognized. Experiments with seeds treated for non-damaging time periods have not been completed.

Procedure for Establishment of Germ Free Pinus palustris and Pinus elliotti

Germ free plants are required for challenge of quarantined materials from lunar missions and for studies of effects of lunar minerals upon plant growth. The following procedure was developed for producing and certifying germ free plants of Pinus palustris and Pinus elliotti.

There were three distinct phases in the procedure.

(1) Sterilization and germination of seed.

(2) Initial certification and transfer.

(3) Second certification and final transfer.

All operations are carried out in a laminar flow bench or where possible in an axenic botanical chamber (ABC), a plexiglass glove box fitted with a positive pressure and recirculating filtered air system. The ABC is sterilized by fumigation with heated paraformaldehyde.

Sterilization and Germination of Seed

Under the cleanest possible conditions (i.e. squeaky clean hands and instruments) the seed coats were removed from the pine seeds. The seeds were then soaked in a solution of phosphate buffered 0.5% NaOCl (as

prescribed in NASA Manual MSC03267) for six minutes. Following this the seeds were rinsed three times in sterile distilled water with a contact period of two minutes per rinse.

Sterilized seeds were aseptically introduced, one seed each into the primary container on Whites S-3 medium with sucrose. The primary container consisted of a 5 dram wide mouth Virtis vial half filled with Whites agar and a 22x60 mm paper extraction thimble filled with a mixture of 65% sand and 35% perlite, both contained in a capped 4 ounce Texberry jar. This assembly was sterilized by autoclaving the Texberry jar containing filled thimble and empty vial for four hours at 130°C, then adding the Whites medium to the vial and autoclaving again for 20 minutes.

Initial Certification and Transfer

After germination of the seed and before root development had progressed appreciably, the vial was filled with sterile trypticase soy broth (TSB) and the perlite-sand in the filter moistened with 5-7 ml of sterile Hoagland's medium. The seedling was then aseptically removed from the vial and buried in the perlite-sand. All of this was accomplished with the appropriate aseptic precautions. A newly sterilized piece of polypropylene was used to cover the jar. While the seedling continued to grow in perlite-sand, the vial containing the TSB was observed for development of microorganisms indicating contamination of the seedling. If the seedling remained healthy and the vial free of apparent contamination for one month, it was considered to have passed the first certification.

Second Certification and Final Transfer

Following completion of the first certification, the thimble containing perlite-sand and seedling was carefully transferred to a sterile 50 ml

beaker contained in a quart fruit jar and covered with sterile TSB. The plant was left in the TSB for 3-4 days to allow time for outgrowth of possible contaminating organisms. If the broth remained clear the second certification was completed and the plant was ready for transfer to its final growth container. The final growth container was a one pint mason jar filled with perlite-sand and fitted with four vials containing different growth media (TSB, potato dextrose broth, thioglycollate broth and Bristols medium with cellulose). Vials were spaced around the rim with mouths level with, or just above, the perlite. Jar, perlite-sand, and empty vials were sterilized at 220°C (dry heat) for five days, then media were added and autoclaved. When fully assembled the growth container was capped with a 12 inch high teflon bag held in place by the outer screw ring of the jar. The teflon bag had a 4 inch airway sealed along one side with a heated bag sealer. This airway was plugged with glass wool. Sterilization was accomplished with the teflon bag wrapped in a paper bag. Autoclaving was by a 2 hour and 45 minute cycle which included a 15 minute prevacuum and a 30 minute post vacuum to insure penetration of the steam into the glass wool and drying of the glass wool after sterilization.

For final transfer the seedling was removed from the extraction thimble and the root pushed into a prepared hole in the center of the container. Sixty ml of sterile Hoagland's solution was added to the perlite-sand and the teflon bag was fastened into place. Watering as needed as accomplished by injecting with a sterile syring through the teflon bag, swabbing the injection area with alcohol before and after injection. Growth continued in this unit until the end of the experiment. The included media provided a system for monitoring accidental intrusion of contaminants.

Table 1. Effect of length of sterilization[1/] period upon germination of seeds of twelve lettuce varieties.

Variety	Germination Percentage at One Day in Seeds Treated for Periods of 15 min.	10 min.	7.5 min.	5 min.	2.5 min.
Imperial 847	53	51	69	78	
Paris Island	0	2	20	68	84
Great Lakes	0	0	14	50	
Burpees Iceberg	17	45	53	85	
Dark Green Boston	3	85	95	96	
Salad Bowl	89	83	84	91	
Tom Thumb	87	90	96	93	
Grand Rapids	80	85	85	90	
Ford Hook	96	93	96	100	
Mesa 659	0	0	5	43	44
Oak Leaf	75	83	88	96	
Butter Crunch	65	89	85	95	

1/ Sterilizing solution - buffered hypochlorite

Table 2. Effect of length of sterilization[1/] period upon vigor of seedlings of twelve lettuce varieties after six days of growth.

	Period of Sterilization	Vigor Class 2 cm	2 cm-0.5 cm	stunted	ungerm.
Imperial 847	15	57.6	28.8	1.5	12.1
	10	69.7	13.1	9.2	7.9
	7.5	57.3	31.2	6.2	5.2
	5	71.8	19.4	1.9	6.8
Paris Island	10	2.8	44.0	47.7	7.5
	7.5	22.0	55.9	16.5	5.5
	5	45.9	40.4	8.2	5.5
	2.5	72.2	24.7	2.1	1.0
Great Lakes	15	0	0	23.6	76.4
	10	0	32.7	50.5	16.8
	7.5	5.4	72.8	20.6	1.1
	5	9.1	69.3	20.4	1.1
Burpee's Iceberg	15	15.5	31.1	34.9	18.4
	10	36.0	37.8	23.4	2.7
	7.5	54.8	26.0	19.2	0
	5	69.2	26.4	2.2	2.2
Dark Green Boston	15	1.1	3.4	75.9	19.5
	10	53.3	30.4	10.9	5.4
	7.5	85.5	7.8	6.7	0.0
	5	90.7	5.1	2.1	2.1

	Period of Sterilization	Vigor Class 2 cm	2 cm-0.5 cm	stunted	ungerm.
Salad Bowl	15	88.1	10.7	0.0	1.2
	10	82.3	12.9	3.5	1.2
	7.5	87.5	3.1	4.7	4.7
	5	84.6	12.8	1.3	1.3
Tom Thumb	15	84.8	10.5	2.3	2.3
	10	90.0	6.1	1.2	2.4
	7.5	90.0	9.2	1.2	0.0
	5	92.2	5.2	2.6	0.0
Grand Rapids	15	70.7	28.3	0.0	1.0
	10	79.8	19.2	1.0	0.0
	7.5	76.7	20.9	2.3	0.0
	5	82.5	8.7	1.2	7.5
Ford Hook	15	69.7	27.3	3.0	0.0
	10	85.7	10.2	2.0	2.0
	7.5	85.7	11.9	2.2	0.0
	5	85.4	11.2	2.2	0.0
Mesa 659	10	0.0	0.0	27.4	72.6
	7.5	0.0	36.9	27.7	35.4
	5	0.0	91.8	2.0	6.1
	2.5	0.0	78.0	7.3	1.5

	Period of Sterilization	Vigor Class 2 cm	2 cm-0.5 cm	stunted	ungerm.
Oak Leaf	15	6.1	26.1	62.6	5.2
	10	54.0	34.0	11.0	1.0
	7.5	75.0	16.1	7.1	1.8
	5	86.2	12.8	1.1	0.0
Butter Crunch	15	84.5	13.1	2.4	0.0
	10	81.4	10.5	4.6	3.5
	7.5	88.0	9.3	2.7	0.0
	5	91.9	8.1	0.0	0.0

1/ Sterilizing solution - buffered hypochlorite

The following summarizes the progress of Contract NAS 9-12050 for the period September 1 through September 30, 1971. Tasks covered by this report are (1) 1.2.1, a and b of Exhibit A, Statement of Work for the Investigation of the Effects of Lunar Materials Upon Terrestrial Vegetation, and (2) Experiments directed toward development of procedures for carrying out tasks 1.2.2 and 1.2.4 of Exhibit A.

Germination Rates of Disinfected Lettuce Seeds After Storage

Seeds were prepared as indicated in the August report with the exception that treatment time was decreased to prevent damage to the seeds. Storage was in the described storage units at room temperature in the dark. Tables 1 and 2 summarize the results of the storage experiment. Storage for periods up to 6 weeks after treatment in general have apparently no significant effect upon germination percentage of the seeds or vigor of lettuce seedlings.

Effect of Agar Upon Germination and Growth

Fisher brand agar, bacteriological grade, has been used in the standard testing procedures. Other agars have been routinely compared with it. All agars were prepared in the same manner except Noble Special Agar (Difco) which was found too soft at 1.0 percent. A 1.5 percent gel was used. The results of comparisons with two lettuce varieties are given in Table 3. The length of the stem of seedlings seems to be a fair index of vigor of the seedling and the combined contributions of seed reserves and the contribution of the agar. At 6 days, most of the seed reserves have been exhausted. The less purified agars appear to contribute a good deal to growth. There also appear to be components present which inhibit growth and germination of some seeds. The greatest uniformity of

germination and growth was on Difco Purified Agar. It might also be worth considering the use of agarose for critical experiments.

A Simplified Procedure for Surface Sterilization

The currently used procedure for surface sterilization of lettuce seeds (NASA Manual MSC 03267) involves the use of covered beakers into which the disinfectant and washing solutions are successively poured. The procedure requires much hard work and a great deal of preparation since beakers and bottles of sterile water must be prepared for each seed sample. The result is that preparation requires a considerable amount of time and a large expanse of bench space to hold all of the materials for even a simple sterilization experiment. We have developed and tested a simplified system which we have found suitable at least for lettuce seeds.

Components.- Materials required are 5 or 10 ml disposable plastic syringes, a piece of fine mesh nylon netting, a length of 1/8" OD vinyl tubing, a 2 liter filter flask, a one hole rubber stopper to fit the flask and a 1 ml pipette.

Assembly.- The inside of the syringe barrel, but not the plunger, is thoroughly cleaned with chloroform and then a circle of nylon netting is welded to the inside of the closed end using a small heated rod. A short piece of vinyl tubing is pushed over the luer fitting leaving approximately 3/16 inch projecting beyond the luer tip. The plunger is replaced in the barrel.

The pipette is inserted in the rubber stopper and adjusted so that when the flask is tightly stoppered the mouthpiece end of the pipette is

just above the bottom of the flask. The delivery tip should then project approximately 1 inch above the top of the stopper. The flask containing 1500 ml of distilled water is autoclaved for 1 hour. The stopper and pipette tip are covered with aluminum foil and the side arm of the flask is plugged with cotton.

Sterilization Procedure.- The syringe is loaded with 250 mg of seeds (200-250 seeds). The usual solution of hypochlorite is prepared in a 250 ml beaker. Sterilization is initiated by drawing the solution into the syringe and shaking rapidly to wet the seeds. After 1/2 minute the solution is expelled and replaced with fresh solution. Within the following 2 minutes this is repeated four times. Seeds treated longer than 2-5 minutes are given a final change of the solution and allowed to sit until the end of treatment. To rinse the hypochlorite is expelled and distilled water is drawn from the distilled water flask through the projecting pipette tip, the vinyl tubing serving as a sealing collar. Rinsing is repeated 6 times over a period of 10 minutes.

Preparation for Activation Experiments.- Tentative selection of five varieties has been made for purposes of development. Varieties are Tom Thumb, Grand Rapids, Ford Hook, Oak Leaf and Great Lakes. The first four represent the fastest growing most vigorous varieties. The last is possibly the worst of all. It is suggested that possibly more can be learned from testing a poor grower for improvement than from testing fast growers.

Experiments are under way on development of a chamber for testing growth in presence of activated lunar and terrestrial materials. The

several systems undergoing trials are of two basic types. One is based on the system for final transfer and growth of germ free pine seedlings described in the August report. The other system utilizes containers of various configurations inside of larger sterile containers. Current ideas regarding requirements for the test system include the following:

1. Room for growth in a germ-free state for periods up to one and one-half months.
2. Simplicity of sterilization and assembly.
3. Confinement of activated materials to a relatively small area to prevent waste and to allow efficient contact by the plant roots.
4. Inertness in the support medium with minimum contribution to the nutrition of the seeds.
5. Ease of recovery of activated lunar materials at the end of the experiment.

TABLE 1. Percentage germination at two (2) days of seeds of twelve lettuce varieties stored for various times after treatment.

Variety	Percent Germination After Days Storage			
	0 Days	7 Days	14 Days	42 Days
Imperial 847	86	77	88	90
Paris Island	87	96	90	89
Great Lakes	88	91	89	93
Burpees Iceberg	92	95	95	96
Dark Green Boston	84	86	85	89
Salad Bowl	97	98	94	89
Tom Thumb	98	96	94	99
Grand Rapids	99	95	99	95
Ford Hook	95	99	99	95
Mesa 659	85	90	85	74
Oak Leaf	89	91	95	93
Butter Crunch	22	37	65	47

TABLE 2. Final germination and seedling vigor of twelve lettuce varieties stored for various times after treatment.

	Days Storage	Percent Germination at 6 Days	Percent of Seedlings		
			Over 2cm	2cm-0.5cm	Under 0.5cm
Imperial 847	0	89	64	24	1
	7	90	65	17	7
	14	94	69	22	3
	42	93	72	13	7
Paris Island	0	99	62	33	4
	7	99	81	15	2
	14	98	75	22	1
	42	98	74	20	3
Great Lakes	0	99	17	73	9
	7	100	23	66	11
	14	98	-	-	-
	42	100	33	56	12
Burpees Iceberg	0	99	56	37	6
	7	99	62	30	7
	14	99	84	10	5
	42	99	91	4	3
Dark Green Boston	0	96	26	23	46
	7	94	19	54	20
	14	93	21	40	31
	42	95	53	25	17

	Days Storage	Percent Germination at 6 Days	Percent of Seedlings Over 2 cm	2 cm-0.5 cm	Under 0.5 cm
Salad Bowl	0	100	85	12	3
	7	100	87	11	2
	14	100	79	19	1
	42	90	81	6	3
Tom Thumb	0	99	66	29	3
	7	97	80	13	4
	14	100	93	4	3
	42	100	92	7	1
Grand Rapids	0	100	81	16	3
	7	98	85	7	5
	14	100	72	28	0
	42	98	86	10	1
Ford Hook	0	98	73	22	2
	7	100	90	8	2
	14	100	99	1	0
	42	100	94	1	5
Mesa 659	0	96	2	92	2
	7	98	14	83	0
	14	97	21	65	12
	42	98	52	32	14

	Days Storage	Percent Germination at 6 Days	Percent of Seedlings Over 2 cm	2 cm-0.5 cm	Under 0.5 cm
Oak Leaf	0	96	47	35	14
	7	96	45	34	17
	14	0	71	19	9
	42	0	78	11	10
Butter Crunch	0	90	9	53	28
	7	95	11	47	37
	14	96	21	43	31
	42	92	18	34	40

Table 3. Germination and growth of lettuce seeds in various grades of agar.

Agar	Lettuce Variety	Percentage of Seedlings[1] in Length Class: Over 4 cm	4 cm to 2 cm	2 cm to 0.5 cm	Stunted	Ungerm.
Fisher Bacteriological	Ford Hook[2]	71.4	20.6	6.8	0.9	0
	Great Lakes[3]		30.5	55.9	12.6	1.0
Difco Bacteriological	Ford Hook	24.8	66.5	5.0	3.0	0.5
	Great Lakes		16.1	67.2	14.9	1.5
Noble Special Agar	Ford Hook	14.4	82.2	1.9	2.14	0
	Great Lakes		15.1	61.9	21.2	1.8
Oxoid Ionagar #2	Ford Hook	2.0	92.9	2.4	2.3	0
	Great Lakes		29.4	57.0	9.75	1.5
Difco Purified	Ford Hook	0.3	94.6	3.1	1.6	0
	Great Lakes	0.0	20.9	70.5	8.2	0.6

1/ Average of 8 plates tested with 50 seeds per plate. Counts made at 6 days.

2/ Disinfection time was 5 minutes.

3/ Disinfection time was 2.5 minutes.

The following summarizes the progress of contract NAS9-12050 for the period January 1 through January 31, 1972.

Experiments are continuing in an effort to confirm the germ-free condition of lettuce seeds. Higher percentages of contamination are observed when seeds are allowed to germinate and grow for 2-4 days on purified agar prior to plating on a nutrient agar. Contaminating organisms (all bacteria) appear to be largely associated with the stem and cotyledons rather than with the roots. Killing of seeds or seedlings by freezing prior to plating has no effect upon normally observed contamination. In no case has contamination been above 5 percent; thus lettuce at this point still appears to be a relatively clean species.

Preliminary experiments have been completed with the growth chambers for exposure of lettuce seedlings to neutron activated solid materials. Simulated lunar or lunar dust will be mixed with dampened sand which will serve as the support medium for germinating seeds or will be spread over the roots of seedlings already growing on a thin layer of sand in the standard growth chambers submitted previously. Upon the advice of members of the Nuclear Science Center, staff activation times will be shortened to one to a few days and exposures will be no more than 1 week.

The following is a periodic progress report summarizing the most recent results of work done under contract NAS 9-12050.

One portion of this project has been concerned with the use of tissue culture as a means of evaluating the effects of lunar or terrestrial mineral material upon cell growth. Experiments were done with two main objectives in mind. One objective was to learn something about the properties of the tissue and conditions giving optimal growth. The other was to decide if tissue culture could actually be used to study uptake of elements from simulated lunar soil. Lettuce was used as the test tissue.

PART 1

Growth and Properties of Lettuce Tissue Culture

Materials and Methods. Initially five varieties were tested to determine the most suitable for these experiments. These were:

7	Tom Thumb
8	Grand Rapids
9	Ford Hook
11	Oak Leaf
17	Prize Head

Variety Oak Leaf was selected and all of these experiments were done with it.

Explants were from seeds sterilized as described in an earlier report and planted on agar prepared in Hoagland's #2 solution. Seedlings were allowed to grow 5-15 days before use. Whole leaves or parts of leaves or roots were used. All plant parts were transferred to the surface of the tissue culture medium using aseptic technique but without further surface sterilization.

Media used were Murashige and Skoogs medium (MS) unmodified and Murashige and Skoogs medium as modified by S. Venketeswaran (SV medium). SV medium was tested with various components increased, decreased or deleted and with additional supplementation. The basic SV and MS media are described in the NASA Protocol manual MSC 03267. SV medium was employed in limited tests of liquid culture. Major salts were tested in various dilutions. Trace elements and vitamins were used in normal concentration. Solid medium was prepared with 1% purified Difco agar. Plastic 15 by 100 mm sterile disposable petri dishes were used for all experiments with growth on solid medium. Edges were sealed with scotch tape to retard desiccation.

Formation of callus was allowed to progress for 4-6 weeks at 24C under fluorescent light. Growth was evaluated by fresh weight and in some experiments by Lowry total protein and acid phosphatase.

Results and Discussion. The following general observations may be made conderning tissue culture:

Lettuce is an extremely easy tissue to culture. Even in a medium devoid of complex additives such as coconut milk, callus begins to form on the explant at 5 to 7 days. In our case this was probably partly traceable to the fact that plants were grown from sterilized seeds and thus surface sterilization which could retard outgrowth was not necessary.

Rapidity of callus outgrowth appears to be independent of plant parts (roots, leaves, stems), provided a cut surface is exposed; of age of plant (within the limits of 5-15 days); of growth conditions (light, dark, or temperature from 20-30C); or of variety (for the five varieties tested).

Fresh weight yield, though probably not physiological condition, was not significantly different at growth temperatures from 20 to 30C.

Growth in liquid medium may be initiated from either callus or fresh

plant parts. The best growth is observed in SV medium containing 1/2 to 1/4 the concentration of major salts employed in the standard solid medium. Shaking of cultures on a rotary shaker results in a high number of broken cells and much cell debris in the culture. Culture in tubes on a roller apparatus appears to be more practical method of growing liquid cultures.

Various experiments were carried out with conditions of growth of lettuce callus cultures. The following conditions were common to all experiments:

SV basal consisted of all salts hormones and vitamins of SV medium without the complex additives coconut milk and yeast extract and without ascorbic acid.

All proportions given are fractions or multiples of the amounts specified in the protocol manual.

All explants were parts of leaves ranging in weight from 7 to 12 mg.

The following experiments were done:

Effect of pH on growth of tissue explants of several varieties of lettuce using complete SV medium prepared and adjusted before autoclaving to pH 4.8, 6.0 or 7.5. Culture age 1 month

Variety	Grams fresh weight yield		
	pH 4.8	pH 6.0	pH 7.5
7	2.411	3.010	3.112
8	2.775	2.600	2.407
9	1.943	2.217	2.111
11	3.556	3.722	3.461
17	2.247	2.351	2.014

Effect of varying amounts of yeast extract upon growth. Culture age 6 weeks.

Treatment	Grams fresh weight yield
SV coconut- yeast	2.469 ± .622
SV coconut + 1/4 yeast	3.072 ± .297
SV coconut + 1 yeast	3.356 ±1.495
SV coconut + 2 yeast	3.584 ±0.731

Effects of varying amounts of ascorbic acid upon growth. Culture age 4 weeks.

SV basal - ascorbic	2.802 ± .842
SV basal + 1/4 ascorbic	2.682 ± .654
SV basal + 1/2 ascorbic	2.756 ± .583
SV basal + 1 ascorbic	2.977 ±1.042
SV basal + 2 ascorbic	2.323 ± .644

Effect of varying amounts of coconut milk upon growth. Culture age 7 weeks

SV basal	4.031 ± 1.000
SV basal + 1/4 coconut	4.965 ± 1.492
SV basal + 1/2 coconut	4.287 ± .558
SV basal + 1 coconut	5.375 ± 1.402
SV basal + 2 coconut	7.375 ± 2.242

Effect of varying amounts of KH_2PO_4 upon growth. Culture age 4 weeks

Treatment	Grams fresh weight yield
SV basal - KH_2PO_4	.041 ± .022
SV basal + 1/4 KH_2PO_4	.571 ± .426
SV basal + 1/2 KH_2PO_4	1.362 ± .573
SV basal + 1 KH_2PO_4	1.836 ± .625
SV basal + 2 KH_2PO_4	2.418 ±1.293

Effect of varying amounts of $CaCl_2$ upon growth. Culture age 6 weeks

SV basal - $CaCl_2$	3.170 ± .558
SV basal + 1/4 $CaCl_2$	2.310 ± .911
SV basal + 1/2 $CaCl_2$	2.592 ± .974
SV basal + 1 $CaCl_2$	2.286 ± .557
SV basal + 2 $CaCl_2$	1.561 ± .625

Effect of varying amounts of $MgSO_4$ upon growth. Culture age 6 weeks

SV basal - $MgSO_4$	.574 ± .359
SV basal + 1/4 $MgSO_4$	3.214 ± .480
SV basal + 1/2 $MgSO_4$	2.089 ± .623
SV basal + 1 $MgSO_4$	2.924 ± .799
SV basal + 2 $MgSO_4$	1.939 ± .993

Effect of various vitamin additives upon growth. Culture age 5 weeks

Treatment	Grams fresh weight yield
SV basal	3.183 ± 1.152
SV basal + myo inositol	2.066 ± .534
SV basal + Ca pantothenate	2.831 ± .791
SV basal + B_2	3.397 ± 1.132
SV basal + Ca pantothenate + B_2	4.253 ± 1.100
SV basal + Ca pantothenate + myo inositol	3.594 ± .858
SV basal + B_2 + myo inositol	4.295 ± .540

Experiments with handling of tissue and its effects upon enzyme (acid phosphatase) activity. Effects of homogenization in buffer (0.005 M. Tris-maleate pH 6, + .001 M $MgSO_4$ and 1 mg/ml Sodium Ascorbate) compared to homogenization in distilled water. Culture age, 4 weeks. Homogenates prepared from whole callus after storage for 4 days at 4C.

Treatment	Homogenate	Enzyme Units /mg Protein
SV basal + 2 $CaCl_2$	Water	0.268
SV basal + 2 $CaCl_2$	Buffer	0.346
SV basal + 1 $CaCl_2$	Water	0.369
SV basal + 1 $CaCl_2$	Buffer	0.411
SV basal + 1/2 $CaCl_2$	Water	0.309
SV basal + 1/2 $CaCl_2$	Buffer	0.421
SV basal + 1/4 $CaCl_2$	Water	0.388
SV basal + 1/4 $CaCl_2$	Buffer	0.437

Total Lowry protein and acid phosphatase activity of lettuce callus tissue of different ages after growth in the presence of varying levels of added calicum.

Treatment	Age	Total Protein mg/g fresh wt	Acid Phosphatase Enzyme Units/mg protein
2 Calcium	Dark	0.496	2.089
	Medium	0.715	1.222
	Light	0.778	1.154
1/2 Calcium	Dark	0.558	1.870
	Medium	0.678	1.445
	Light	1.154	0.809
1/4 Calcium	Dark	0.558	1.709
	Medium	0.665	1.412
	Light	1.079	0.793
0-Calcium	Dark	0.828	1.042
	Medium	1.154	0.742
	Light	1.286	0.701

Total Lowry protein and acid phosphatase activity of lettuce callus tissue of different ages after growth in the presence of varying levels of added magnesium.

Treatment	Age	Total Protein mg/g fresh wt	Acid Phosphatase Enzyme Units/mg protein
2 Magnesium	Dark	0.857	0.934
	Light	1.142	0.635
1 Magnesium	Dark	0.774	1.417
	Light	1.964	0.538
1/2 Magnesium	Dark	0.815	1.208
	Light	1.577	0.690
1/4 Magnesium	Dark	0.892	0.777
	Light	1.232	0.517
0-Magnesium	Total	1.756	0.214

PART 2

Use of Tissue Culture for Studying Uptake of Minerals from Simulated Lunar Soil

Materials and Methods. Media and methods of cultivation were identical to those outlined in part 1. Simulated lunar soil lot #005 was weighed in varying amounts into capped polypropylene tubes and autoclaved at 1 psi for twenty minutes. Prior to pouring of the medium (20 ml/plate) the contents of individual tubes were emptied into the plates and mixed into the ungelled medium by gentle swirling. Leaf explants averaging 10 mg were planted on the solidified agar at the rate of 3 explants per plate.

Results and Discussion. The following general conclusions can be made concerning the use of tissue culture for assaying the effects of mineral elements from simulated lunar soil or other mineral samples:

The method used here is a very sensitive one for showing that soluble materials are reaching the tissue explant.

The effects found (inhibiting) are very probably caused by excess toxic materials or competition of non-essential elements with essential elements rather than pH changes because previously pH has been found to have relatively little effect over a wide range.

Any stimulatory effect of the simulated lunar soil will probably appear at concentrations below those used in these experiments. If this is the case, the method is much less wasteful of mineral materials than methods in which pre-grown callus is treated with mineral materials.

Measurements of uptake of minerals will be difficult in this system because of the relatively small growth obtainable from the

tissue and also because of the apparently large background of minerals contributed by the purified agar.

The following experiments have been done:

Effect of varying amounts of simulated lunar soil batch #005 upon growth of lettuce explants. Culture age 4 weeks

Variety	Grams fresh weight yield			
	SV basal	+100 mg	+500 mg	+2000 mg
8	1.240	.267	.027	.016
9	1.005	.206	.070	.053
11	1.115	.406	.097	.041
17	.945	.162	.105	.074

Effect of varying amounts of simulated lunar soil upon pH of 20 ml of SV basal medium.

Time after addition	pH			
	0	50 mg SL	500 mg SL	2000 mg SL
1 hour	5.40	5.41	5.98	6.52
2 Days	5.41	5.50	6.10	6.78

pH of 20 ml of distilled water or 1 percent purified agar suspensions of various amounts of Simulated Lunar soil.

Medium	pH			
	0	50 mg SL	500 mg SL	2000 mg SL
Water + SL #005	6.70	6.92	8.65	9.50
Water + SL #002	6.70	6.85	8.75	9.56
Difco Purified Agar + SL #002	5.80	6.76	7.40	8.33

The following summarizes the progress of contract NAS9-12050 for the period June 1 through June 30, 1972.

Experiments with the ability of powdered basalt (simulated lunar soil) to support plant growth are continuing. The results of a large number of trials with lettuce growth in various types of apparatus under different growth conditions indicate three main conditions to be met. First, in any appreciable volume of the simulated lunar material it is necessary to "fluff" the powder to allow aeration of the plant roots. In experiments with small amounts of powdered basalt and small plants, this has been solved by growing the plants on a gauze support over the basalt in water, by mixing with acid-washed sand or by sandwiching the powder between rolled layers of washed filter paper. Examples of the various apparatus used have been submitted previously. With volumes of material adequate for growth of a plant to maturity, such systems are not suitable because the samples of simulated lunar material provided are fine powders formulated to duplicate chemical composition but not physical consistency of lunar soil. The lunar soil approaches a sandy loam in general consistency. which may allow adequate ventilation. For tests of this, it will be more important to duplicate the physical properties than chemical, and the preparation of a quantity of such material should be given high priority. For the simulated lunar material currently available we are experimenting with the use of polyurethane foam and Dacron fiber as bulking agents.

A second requirement is for supplementation with nitrogen and to some extent phosphorus. This has been accomplished thus far by addition of the salts in solution, however, under these conditions the utilization of nitrate and phosphate leaving behind the sodium potassium or calcium results in a rise in pH. A third requirement is, therefore, the maintainence of a reasonable pH. We are attempting to solve both requirements by constant leaching of the soil with dilute ($<$.001M) HNO_3 and H_3PO_4. Results thus far have been encouraging, although the requirement for germ-free conditions greatly complicates the mechanics of the system.

Tissue culture of lettuce has been accomplished with a minimum of difficulty. Callus formation is evident after 4 to 5 days in SV medium (NASA Protocol Manual). Growth rate is good though not spectacular. Tissues are tan to brown indicating a considerable amount of pigment synthesis, probably tannins. There is no noticable difference in growth when coconut milk is excluded from the medium and only a moderate reduction in growth with the exclusion of yeast extract. We are in the process of optimizing the various components of the medium for growth of lettuce.

Callus is initiated most easily on agar using short sections of stem tissue. Leaves are resistant and must be cut and pressed into the agar. Plants used in these experiments are grown 6 days from surface sterilized seeds planted in Hoaglands agar (1% agar in Hoaglands #2 solution). Of the five varieties we have cultured, Tom Thumb, Grand Rapids, Ford Hook, Oak Leaf, Prize Head, relatively little difference has

been apparent in rate or appearance. We have thus used only Oak Leaf for the experimentation.

Liquid shaken cultures may be readily initiated in SV medium (minus coconut milk) using either plant parts or pre-grown callus. Leaves or pre-grown callus are better than stems. Leaves tend to produce strings of cells under the conditions currently being used, however, a second generation of well suspended cells may be grown by re-inoculating with only the suspended portion of the leaf cell culture. Presently, we use 50 ml of medium shaken at 150 rpm in a standard 250 ml erlenmeyer flask. We are also experimenting with growth in an aerated culture system which we have employed in the past for growing fungus cultures.

The following summarizes the progress of contract NAS9-12050 for the period July 1 to July 31, 1972.

Experiments with lettuce seed on various amounts of Simulated Lunar soil (SL) mixed with 1% purified agar have suggested significantly increased germination rate and increased rate of elongation in the presence of SL soil (Table 1). Growth of lettuce seedlings in the presence of SL soil is, in general, decreased as amounts of soil in contact with the seedlings are increased above a few hundred milligrams per growth chamber (Table 2). These experiments were carried out in the folded filter paper growth vessels in polypropylene covered quart mason jars.

These results were not completely unexpected and this expectation was in part the basis for our earlier decision to begin some practical experiments with leaching treatments of SL soil. It seems likely that SL soil (general conclusions can probably be extended to lunar soil although supporting data are not available) provides limited amounts of mineral nutrients which are beneficial to germination and to elongation of the hypocotyl. Inhibition appears to result from the release of excessive amounts of mineral elements by the action of the growing plant roots. Whether the mechanism of inhibition is in release and subsequent uptake of toxic elements or by competition and exclusion of essential elements by non-essential elements we cannot say at this point. Analysis of the tissue harvested from these experiments should provide some answers.

Table 1. Effects of simulated lunar soil upon germination of seeds of oak leaf lettuce.

Simulated lunar soil, mg	Average percent germination at 24 hrs	48 hrs	144 hrs	Average hypocotyl length at 144 hrs
0	42.0	84.8	95.0	2.54
50	69.9	90.6	97.0	2.33
100	66.9	88.8	97.9	2.68
200	66.8	90.3	96.3	2.68
500	70.5	91.8	97.5	2.58
1000	67.6	90.6	97.0	3.08
2000	72.6	92.6	96.4	3.45

Table 2. Growth of germ free lettuce plants of five varieties in the presence of varying amounts of simulated lunar soil.

Simulated lunar soil, mg	Average dry weight yield[a] per growth chamber #7	#8	#9	#11	#17
0	7.78	11.73	14.45	38.76	10.52
100	25.00	10.20	16.12	42.17	5.08
500	13.02	10.12	16.13	42.08	6.34
1000	12.48	13.00	12.74	39.12	9.03
2000	16.15	10.28	16.12	35.84	3.94

[a]Three seedlings per chamber after 5 weeks growth.

The following is a periodic progress report summarizing the most recent results of work done under contract NAS9-12050.

In addition to measurement of mineral elements taken up into lettuce plants growing on various soils, a portion of this project has been concerned with the release of elements from simulated lunar soil and other materials by the action of lettuce roots or root exudates and microorganisms.

General Method

The general approach employed involves the growth of the plants or microogranisms or both together upon known amounts of the mineral substrate followed by collection of the solute and analysis of it to determine minerals released. Minerals taken up into the upper plant parts are also measured in accordance with the primary objectives of the project. Roots are excluded however because of the impossibility of separating them from the solid mineral substrate. Three separate types of experiments are being done: release by microorganisms alone and in various combinations on a variety of organic substrates, release by action of plant roots under germ free conditions, and release by confined action of plant roots and various microorganisms or combinations of microorganisms.

Release by Microorganisms

One hundred milligram samples of the mineral to be tested are weighed into clean polypropylene culture tubes (Falcon 2006) and autoclaved 15 minutes at 15 psi. To these tubes are added 0.5 ml of sterile distilled water or nitrogen-phosphorus salts solution containing various concentrations of organic substrates. Insoluble substrates are added with the rock. The microbial inoculum is suspended in distilled

water and added to the tubes with a wire loop. After incubation for a period of time, usually one month, 2 ml volumes of distilled water are added and mixed on a vortex mixer and solutes are collected and concentrated by migration up a paper wick.

Release by Plants or Combinations of Plants and Microorganisms.

Plants are grown by the gauze method over 500 to 20000 mg of mineral sample with 15 ml basal salts solution. The salts solution consists of 5 ml of distilled water added prior to autoclaving and 10 ml of double strength Hoagland's #2 solution macro elements. If addition of microorganisms is required these are introduced as described above. At the end of the growth period plants are dried and weighed. Solutes are collected as above by migration up a filter paper wick. Given below is the procedure used.

Collection and concentration of Solutes on Filter Paper.

General: The method involves migration of the solution up a strip of filter paper and evaporation of the water leaving the solutes concentrated at the tip. The tip is the cut off and analyzed by an appropriate method. Whatman #42 Ashless Paper is used.

Procedure:

1. Cut Whatman #42 ashless filter paper into 1" x 4" strips.
2. Prewash, insert each strip into a clean Falcon #2006 plastic tube, add 5 ml of double distilled water and allow the water to evaporate.
3. When the strips are dry, trim the top 1/4 inch from the strip and discard. Store the strips in a clean polyethylene bag. At this stage either handle only by the tube or use polyethylene gloves to prevent contamination by minerals on the fingers.

4. To collect solutes insert papers to the bottoms of the experimental vessels and allow to migrate and evaporate to dryness. For plant growth chambers, slit the covering polypropylene film near the edge of the vessel and insert the paper through the slit.

5. Wash by adding distilled water (2 ml for tubes 5 ml for beakers) 3 times allowing the paper to dry between additions.

6. After the final washing, cut off the top 1/2 inch of the strip containing the concentrated solutes and store in a 3/5 dram snap cap polyethylene vial.

7. The storage vial is also the vial used for holding the samples during activation. Such vials should be rinsed carefully with distilled water before use and all labelling should be done by scratching the plastic with a sharp pen. Marks-A-Lot or Magic-Marker ink contains significant amounts of metal contaminants.

Discussion

This line of experimentation is being pursued in order to provide comparative data regarding the actual amounts of soluble minerals available for uptake by the plants. It should also provide some insight into the problems which will accompany attempts to establish an agricultural operation in a lunar base or upon similar terrestrial sites such as fresh volcanic ash falls, eroded areas or areas denuded by mining operations. The purpose of the present report is to outline the experiments and the methods used. A number of samples have been

generated and have been submitted for analysis. Other experiments are in progress. Results of these will be submitted in a future report.

The following is a periodic progress report summarizing the most recent results of work done under contract NAS9-12050.

The primary objective of this project has been to produce lettuce tissue which has been grown under germ free conditions in contact with various mineral substrates. Because of these restrictions, conditions are optimal neither for maximum growth rate nor for production of large amounts of tissue. Nonetheless, a secondary objective has been to draw some conclusions concerning the nutritional value of unweathered minerals to the test plants under the specified conditions.

Methods.

The plants were grown and exposed to simulated lunar soil batch #002 by the filter paper method described previously. Plants were grown for one month ± three days, harvested, dried at 75C over night and weighed to determine yield per treatment.

Results and Discussion

The necessity for minimizing handling and contamination of plants precluded the possibility of obtaining individual weights and thus of calculation of statistics of variability from the data. The cumulative averages are taken, however, from five separate experiments, each having six replications, therefore some valid trends should be recognizable. Table 1 summarizes the yield data for SL soil.

There is in general an increase in yield from plants treated with SL soil. This is probably significant. In addition there appears to be some inhibition of growth at higher concentrations. It is unlikely that this is traceable to differences in pH which is 5.15,

5.42, 5.82, 5.95 and 6.14 for the standard volume of Hoagland's solution containing 0, 100, 500, 1000, and 2000 mg of SL#002 respectively. Final average pH after growth of plants for one month is 4.7, 5.0, 6.2 and 7.4 for Hoagland's solution containing 00, 500 and 2000 mg of SL #002 respectively. These latter values are taken from experiments with growth in the gauze chamber. In all cases the SL treated plants have been noticably greener in color at all treatment levels. Color photos recording this are on file at the Manned Spacecraft Center.

Table 1. Dry weight yield in milligrams per chamber for five lettuce varieties grown in the presence of varying amounts of simulated lunar soil batch #002

Milligrams SL soil	Lettuce Variety 7	8	9	11	17
0	11.40	9.37	13.12	13.41	8.61
100	14.37	10.39	14.73	15.50	7.35
500	13.65	9.86	14.50	15.59	6.48
1000	12.08	10.25	13.97	15.87	8.78
2000	12.65	11.07	13.16	14.46	6.65

The following is a periodic progress report summarizing the most recent results of work done under contract NAS9-12050.

The growth experiment employing Apollo-11 soil and simulated lunar soil as described in the May report was completed. After 30 days plants were harvested aseptically in a laminar flow hood. Dry weight yields were taken for each individual growth chamber following drying of the plants overnight at 75 C. Pooled yields for each treatment were stored in plastic vials and given to the center for trace characterization (C.T.C.) for neutron activation analysis.

Yield data are as follows:

Vial Number*	Treatment	Number of Chambers	Total mg yield/chamber
198	Control	9	16.2±2.90
199	Lunar	9	16.5±2.45
200	Sim Lunar	10	15.7±3.46

* Labeled and given to CTC

Growth chambers were prepared for a second cycle. Following harvest chambers were resealed and allowed to stand 4 days to allow time for development of possible contaminating organisms. Ten ml of additional basal salts were added to the top of the sand of each vessel. Surface sterilized seeds were planted and chambers were replaced under the conditions described previously.

May 1 - 31, 1973

The following is a periodic progress report summarizing the most recent results of work down under contract NAS9-12050.

An experiment was initiated to measure uptake of mineral elements for Apollo 11 fines by seedlings of lettuce growing under germ free conditions

Methods

Seeds of lettuce (variety Oak Leaf) were sterilized 5 minutes in buffered hypochlorite by the method described earlier (report Oct. 1, 1971). The growth vessel was the sand type prepared as described (report March 1, 1972; April 1-31, 1973) with 25 ml of acid washed sand in the upper part of the vessel and 20 ml of double strength Hoaglands #2 major salts in the lower part. Following sterilization, the samples of lunar soil or simulated lunar soil (0.47 g per vessel) were spread on the top of the sand and covered with an additional 5 ml of sterile sand. Seeds (3 per chamber) were planted in the top layer of the sand. A sterile 5.5 cm circle of Whatman #1 filter paper having a 2 cm hole in the center was centered over the planted seeds to help protect the seedling leaves from contact with soil or sand during subsequent growth. The planted vessels were placed in quart mason jar growth chambers (report April 1-31, 1973) and placed under the light bank in building 266 at Johnson Space Center. The growth experiment consisted of a total of 30 growth chambers, 10 each of which contained sand alone, sand plus simulated lunar batch #002 or Apollo 11 fines.

The plants will be harvested (report Febr. 1 - March 31, 1973) after growth for 4 weeks and subjected to neutron activation analysis at the TAMU Center for Trace characterization.

The following is a periodic progress report summarizing the most recent results of work done under contract NAS9-12050.

The growth experiment employing Apollo-11 soil and simulated lunar soil as described in the May report was completed. After 30 days plants were harvested aseptically in a laminar flow hood. Dry weight yields were taken for each individual growth chamber following drying of the plants overnight at 75 C. Pooled yields for each treatment were stored in plastic vials and given to the center for trace characterization (C.T.C.) for neutron activation analysis.

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* Labeled and given to CTC

Growth chambers were prepared for a second cycle. Following harvest chambers were resealed and allowed to stand 4 days to allow time for development of possible contaminating organisms. Ten ml of additional basal salts were added to the top of the sand of each vessel. Surface sterilized seeds were planted and chambers were replaced under the conditions described previously.

The following summarizes the progress of contract NAS 9-12050 for the period November 1 through November 30, 1971.

Development was completed on a growth vessel for exposure of plants to neutron-activated lunar and terrestrial materials. An example has been submitted to the Manned Spacecraft Center, Preventive Medicine Division.

Tests are being made of various inert media for dilution of activated materials and support of plants during exposure. Materials being tested singly and in various combinations are washed quartz sand, glass of microbeads various sizes, and fumed silica.

The following summarizes the progress of Contract NAS 9-12050 for the period February 1 through February 29, 1972.

An initial trial measurement of uptake of radioactive elements from a neutron activated sample of simulated lunar material is under way. Preliminary results and conclusions are given below. Notes on details of the experiment and bits of information of possible use to other workers attempting similar experiments are given in an Appendix.

Conditions of the experiment were arbitrarily set, but were based on the results of an earlier preliminary experiment with lunar materials which indicated that short activation times and short periods of exposure were required to allow detection of short half-life isotopes. Conditions of the experiment were set to aid in selection of more nearly optimal activation, cooling and exposure times for future experiments. The use of sand as a dispersion medium for the activated materials was expected to be an improvement over the agar used in the preliminary trial.

Uptake was relatively low. Counts of 100 minutes were required in order to obtain detectable peaks of activity. Uptake of a large number of elements was apparent although the peaks for many of them were just above the lower limits of detectability. With the exception of Na-24 few of the prominent gamma spectrum peaks appear to correspond to elements known to be present from spectrographic analysis of the simulated lunar sample. The spectrum peaks for Mn, Co and Sc, the only elements detected in a preliminary study with activated lunar materials, were either absent or relatively small. Further analysis of decay rates will be necessary for

positive identification but energy peaks for Iridium, Actinium, Terbium, Tin, Rhenium, Tantalum, Samorium, Gallium and Gadolinium appear to be present in lettuce plant tissues after growth for 1-2 days on the activated simulated lunar materials. Prominence of an energy peak is not necessarily correlated with abundance of the element since such factors as ease of activation and the efficiency of the detector for different energy levels have a great deal of influence upon apparent activity. Nonetheless, it is evident that we will have to consider possible effects of some of the less common elements in plants.

The relatively low uptake of activated materials appears to be the main problem to be solved. The standard growth chamber using a sand support for roots and as a dispersing medium for the activated materials will probably require modification. Trials are being made with a mesh support for root growth which will allow roots to be placed directly in contact with activated material suspended in a small amount of water or nutrient solution.

Exposure times must be shortened to allow measurement of short-half-life materials. Relatively old plantlets (1-3 weeks) will be necessary. Germinating seeds, which require several days to produce adequate volumes of material, appear to be unsuitable.

Activation times in future experiments will be decreased probably to 1 to 4 hours. The 12 hr activation time employed in the first experiment resulted in materials requiring an excessively long cooling period. Much of the activity of short-half-life isotopes was lost in the 3-4 days between completion of activation and the first measurements of uptake into the plants.

APPENDIX 1

Notes-February 21, Activation Experiment

Growth and exposure unit was the standard polypropylene apparatus with the following components:

20 ml Knops solution in the lower chamber

a 1 inch Whatman #1 filter paper wick leading from bottom to top chamber.

a 4.5 cm disc of Whatman #1 filter paper covering the bottom of the upper chamber and holding the wick in place.

5 ml of acid washed ottawa sand in the upper chamber as a growth support for the seeds.

in one set of chambers sand was not used and seeds were germinated directly on the filter paper disc.

each growth apparatus was autoclaved in a 250 ml plastic beaker covered with aluminum foil; with a sterilization time of 20 min.

subsequent to seeding, the aluminum covering of the outer 250 ml beaker was replaced with a square of sterile polypropylene film.

Treatments were:

1. Seedlings of variety #8 pre-grown on sand for 8 days $\pm$ 2 hr prior to addition of activated material and harvested for analysis at various times afterwards.
2. Seedlings of variety #7 treated as in 1.
3. Seedlings of variety #8 pre-grown in filter paper for 3 weeks prior to addition of activated material.

4. Seeds of variety #8 germinated and grown for 6 days on activated materials.

5. Seeds of variety #7 germinated and grown as in 4.

10 seeds were planted in a circle in the center of each vessel. The activated material mixed with sand was poured into the center of the circle. All growth was at 24° C with a 12 hour light-dark cycle.

Activation of Simulated Lunar. - Simulated lunar sample #.005 weighed out as a 2 g sample.

Sample was taken to Jack Shannon-chemistry glass shop - who enclosed it in a 9 mm x 4 inch vycor glass tube. This left about 2 inches of open space above the sample. The glass was scored with a diamond pencil about 1/2 inch above the sample to allow breaking of the vial after activation.

Activation for 12 hrs, 5 min. at 1 MW power. Completion at 9:00 p.m., February 21.

Sample was allowed to cool in the capsule until 10:00 a.m., February 23. At this time activity at surface of vial read 4 r/hr.

Vial was broken at the scored point by inserting the bottom in a 3/8" hole bored in a 6" length of 2 x 4 board and applying pressure to the portion above the score with a heavy pair of pliers, using a heavy wrapping of Kimwipe to protect from possible pressurized gases.

Activated material had darkened noticably but no problems were encountered in transferring it to a disposable plastic (Falcon) tube.

Activity was too high to allow safe handling of the whole sample. A portion of the sample, 0.254 g (activity 280-300 mr/hr), was weighed by subtraction and added to 0.658 g of cold simulated lunar. The combined

sample was added to 200 ml of dry sand in a 1000 ml plastic beaker. Distilled water (4.4 ml) was added for wetting and the sample-sand mixture was stirred by hand for 7 min.

Measured volumes were dispensed into disposable plastic tubes and into growth chambers by means of a plastic scoop which delivered 4.5 ml of dry sand. Bulking by water resulted in a reduction in the actual dry equivalent of the activated mixture.

Sand-activated mixture was dispensed into 40 tubes and 11 growth chambers giving a total volume of approximately 230 ml of mixture.

Average amount of simulated lunar/ml = 3.965 mg

Average amount/chamber or tube = 17.84 mg

Average amount of activated/chamber = 4.968 mg

All tubes and chambers were autoclaved 20 min.

Contents of 1 tube of sand-activated material per chamber were carefully added to the center of each chamber of pre-grown lettuce plants. Sand was spread under the leaves of the lettuce plants with the aid of a sterile set of forceps. This process was somewhat tedious since the plants were still small and care was necessary to prevent the contamination of leaf surfaces by the activated materials. Twelve chambers of each variety (8 days) and 4 chambers of #8 (3 weeks) were treated.

Seeds of #7 and #8 lettuce varieties were planted in the sterilized sand-activated mixture. Four chambers per variety were planted with 10 seeds per chamber.

Harvest and Counting of Isotopes Taken up by the Plants. - Pre-grown plants were harvested periodically and counted. Germinated seeds were harvested after 6 days.

Plants were picked from the sand medium using forceps, and stems were severed from the roots with a razor blade. Roots were discarded; only the tops were retained for counting. The contents of 3 growth chambers (8 day pre-grown) or 4 chambers (germinated) were pooled for counting. Single chambers of 3 week plants were used. One to three plantlets were set aside from each sample for trials with whole plant autoradiography. Plants were dipped in distilled water to wash off possible clinging activated materials, blotted dry on Labsorb matting and weighed in a tared polyethylene counting vial. To maintain uniform sample counting geometry, leaves were tamped to the bottom of the vial and packed in place with a wadded Kimwipe.

The following summarizes the progress of contract NAS9-12050 for period April 1 through April 30, 1972.

Development of procedures for activation analysis of minerals taken into plants from lunar fines is continuing. Exposure of growing plants to activated mineral material with subsequent gamma ray spectrometry of the plant materials as originally planned has been found to have several limitations. The most notable one is the fact that the combined cooling and exposure times allow complete decay of isotopes of manganese and potassium both of which are of considerable interest. The method is still adequate for the longer lived isotopes. If exposure to lunar materials results in significant uptake of lanthanides and actinides, analyses by this method may be more convenient. Autoradiographic studies of mineral uptake will of necessity require previously activated material although latent images can be expected to reflect only the distribution of isotopes having long half lives.

Our experience indicates that for survey proposes the commonly used methods of activation analysis are more appropriate. Plants grown in contact with unknown and control substrates are harvested and dried, and weighed samples in polyethylene or quartz vials are irradiated and analyzed. By irradiating the same or replicate samples for successively longer periods of time, isotopes having half lives from a few minutes to several months may be measured in the same tissue. A tissue sample of 100-200 mg dry weight appears to be adequate.

The following summarizes the progress of Contract NAS9-12050 for the period May 1 through May 31, 1972.

Preliminary experiments with 1 month plantlets of lettuce, variety Grand Rapids indicate that it is possible to measure the uptake of minerals from simulated lunar soil by activation analysis of the plants following growth on this substrate. A number of experiments on uptake of simulated lunar minerals are in progress. Analysis of plant material from these experiments will be delayed until all plant material has been collected because of the relatively great expense of activating small numbers of samples.

Mineral uptake experiments are of two kinds. In one type, emphasis is merely upon detecting uptake and incorporation of mineral elements. Certain of the experiments with uptake of minerals have been modified in order to provide information on the ability of various basalts and simulated lunar soil to support growth of plants to maturity and the supplementation or treatment required to accomplish this. Plant materials produced in these experiments will be analyzed as usual for mineral content, however, the main emphasis is upon development of methods for growing plants in finely divided rock, or ultimately, lunar fines.

Lettuce is being studied in tissue culture in preparation for experiments on the effects of simulated lunar and lunar soil upon the mineral metabolism of the cells. Cultures are most easily initiated from stem tissues. Leaves of most of the lettuce varieties tested are strongly resistant to undifferentiated growth in either solid or liquid culture with the media employed up to this time. SV medium (NASA manual)

has been the most successful medium. The cure-all components (coconut milk, yeast extract) appear to have little effect upon growth of cultures.

The following is a periodic progress report summarizing the most recent results of work done under contract NAS9-12050.

Samples were given to the Center for Trace Characterization for preliminary analysis. Data from this analysis are presented as scanning counts only in which peaks with background subtracted are printed out. No absolute quantitation is done. This procedure is, however, an inexpensive way of estimating relative amounts of various elements present in comparable samples. The results are given in Table 1. Samples were weighed and sealed in 3/5 dram polyethylene vials. For detection of short half life nuclides, a short activation of 5 minutes in the Nuclear Science Center pneumatic system was followed by counts of 100 to 300 seconds after delays of 3 minutes to 67 minutes. For longer half life nuclides an activation period of 7 hours was used, followed by counts of 33 to 480 minutes after delays from 1.5 to 34 days. The values in the table were calculated from counts at times yielding the lowest average percent error for each element.

There are definite differences in mineral uptake among the various treatments, thus significant amounts of the rocks are either soluble or are solublized by the action of the lettuce plant roots. It seems that the mineral salts solution prepared with reagant grade chemicals also contains a surprising number of trace contaminants. Energy peaks representing a number of other elements were also present in the scans, but were not tabulated because of large counting errors caused by low count rates. Several unidentified peaks were also present. In order to increase accuracy it will be necessary to increase either sample size or activation time.

A tissue sample including the ones scanned above and mineral samples including the basal salts solution have been submitted

to the Center for Trace Characterization for quantitative analysis. Based upon the results obtained above, samples for absolute quantitation will be subjected to three separate activation and counting sequences. The first two will be identical to those previously employed. A third activation will be performed on the samples encased in high purity quartz for long activation periods (5-7 days) to allow detection and quantitation of the long half life elements that are present in very low amounts. Sample size has been doubled by combining two or more identical treatments. The entire activation and counting sequence for a single sample will require approximately three months for completion.

Table 1. Relative concentrations of elements in germ-free lettuce plants grown on Knops solution, simulated lunar soil plus Knops or lunar soil plus Knops and analyzed by activation analysis.[1]

Element	Knops		Sim. Lunar + Knops		Lunar + Knops	
	Percent of Error[2]	Relative Concentration	Percent of Error	Relative Concentration	Percent of Error	Relative Concentration
Cu	10.69	1.00[3]	15.80	0.79	15.23	1.04
Br	10.64	"	21.01	0.48	17.99	0.82
Mn	1.43	"	1.17	1.78	1.15	2.23
Mg	16.07	"	24.72	0.71	21.96	0.81
Na	2.86	"	1.17	2.31	1.36	2.91
K	6.83	"	11.17	0.73	12.21	0.67
Cl	3.46	"	2.91	1.20	3.09	1.41
Al	15.93	"	8.57	2.38	5.33	4.11
Ca	4.84	"	5.01	0.94	4.70	1.07
Hg+Se	15.07	"	31.47	0.42	24.74	0.47
Cr	13.66	:	23.07	0.50	13.82	0.47
Zn	2.38	"	4.30	0.71	3.71	0.77
Co	10.48	"	5.95	3.41	5.73	4.07
Fe	10.57	"	16.75	0.93	14.11	1.25

1 Soil was added to growth chambers at the rate of 200 mg per plant

2 Sample size was 141 mg for SL, 148 mg. for L, and 165.8 mg for Knops

3 Concentration of elements in plants grown on Knops was taken as unity

The following is a periodic progress report summarizing the most recent results of work done under contract NAS9-12050.

Growth of germ-free plants in the presence of lunar or terrestrial mineral materials has been studied in three different types of growth apparatus. These have been described briefly in earlier reports and examples have been submitted. Detailed descriptions and specifications for construction are given below.

General

All three types were constructed from parts of polypropylene beakers (Tripour) of the 100 and 250 ml sizes. Cutting and shaping were accomplished with an electric soldering gun having a flat tip. For maintainance and certification of germ-free condition of test plants, growth vessels were contained within wide mouth quart mason jars. The jars contained as the certification medium 50 ml of nutrient agar (Difco) prepared according to the recommendations of the manufacturer. Jars were closed with a sheet (5" x 5") of sterile polypropylene film held in place with sealing ring.

All mineral samples were supplemented with a basal salts medium consisting of the major elements of Hoaglands Number 2 medium prepared double strength.

Gauze Chamber

In this type of growth vessel plants were supported on a layer of cheese cloth stretched above the mineral sample in a basal salts medium. The roots were able to grow down through the cloth and into contact with the test material. The growth vessel, itself, was capped with a square of polypropylene film. There was no means of certifying germ-free condition of the plants except by culturing

subsequent to harvest.

Components:

1. A 250 ml Tripour beaker with pouring lips removed.
2. The top 1/2 inch of a 100 ml Tripour beaker, lips removed, with two thicknesses of cheese cloth stretched over the top and fastened by pressing into the melted plastic.
3. Mineral samples ranging from 0 to 2 g.
4. 10 ml of basal salts
5. Three seeds of the specified lettuce variety.
6. A 5 inch square of sterile polypropylene film and a rubber band to hold it in place on the beaker.

Preparation:

A mineral sample was weighed into the beaker and the gauze support ring placed on top of it. The assembly was capped with aluminum foil and autoclaved separately in a test tube and poured into the beaker at the time of planting. Surface sterilized seeds were placed on top of the gauze support and the beaker covered with a square of polypropylene. To ensure adequate moisture for germination, beakers were tipped at the time of planting to allow complete wetting of the cloth support and again the next day after planting.

Folded Paper Chamber

In this type of vessel the roots of the plants were allowed to grow into the mineral sample held between layers of a roll of filter paper standing upright in a beaker containing basal salts medium.

Components:

1. A 5" x 6" rectangle of Whatman #1 filter paper treated as follows:

a. Fold across the 5" dimension giving a unit 5" x 3".

b. Roll and insert with the fold downward in a clean 100 ml plastic beaker containing 15 ml of double distilled water.

c. Allow the water to migrate up the paper and evaporate, then trim off the top 1/4 inch of roll to remove the collected contaminants.

d. Approximately half way between the ends of the trimmed edge make 4 1/4 inch deep cuts about 1/4 inch apart and spread apart the cut portions to provide a shelf for subsequent planting of seeds.

2. A 100 ml plastic Tripour beaker with the lips removed.
3. Mineral material in weighed amounts for 0-2 g spread evenly between the folds of the filter paper.
4. 20 ml of basal salts autoclaved separately.
5. 2 surface sterilized seeds of the specified lettuce variety.
6. A quart wide mouth mason jar containing nutrient agar and capped with sterile polypropylene film.

Preparation:

The filter paper roll containing the mineral sample was placed in the 100 ml beaker and the entire assembly autoclaved in a 250 ml beaker capped with aluminum foil. Two seeds were aseptically planted in the notch formed by the cut area of the fold. The sterilized basal salts solution was added to the beaker and the assembly was aseptically transferred to the quart jar certification chamber.

Sand Chamber

The plants were grown in contact with the mineral sample mixed with a

quantity of acid washed sand. The sand was suspended above a reservoir of mineral salts solution. A filter paper wick served to supply moisture and mineral salts as needed. The assembly was contained within a quart jar certification chamber.

Components:

1. A 100 ml plastic beaker with the pouring lips removed and having nested at a level with its top:
   a. A sleeve prepared by cutting another beaker at the 40 ml and 90 ml marks.
   b. A cup prepared from the remainder of the cut beaker below the 40 ml mark. A slit is cut in the bottom of the cup to allow the insertion of a 1 inch wide paper wick.
2. 25 ml of acid washed Ottawa standard testing sand which has been washed at daily intervals over a period of 2 weeks with a total of 2 liters of concentrated reagent grade HCl per liter of sand and subsequently rinsed to neutrality with double distilled water.
3. 5 ml of the same sand sterilized in a separate glass tube.
4. A strip of Whatman #1 filter paper (1 inch x 3 inches) which extends through the slit in the cup to the bottom of the beaker.
5. A 4.25 cm circle of Whatman #1 filter paper placed over the bottom of the cup to retain the wick and prevent loss of sand through the slit.
6. 20 ml of basal salts added by pouring through the sand prior to autoclaving.
7. A weighed sample of the test material in a 13 x 100 mm pyrex

tube capped with aluminum foil and sterilized by heating for 3 hr. at 160 C.

8. 3 surface sterilized seeds of the specified lettuce variety.
9. A quart wide mouth mason jar containing nutrient agar and capped with polypropylene film.

Preparation:

The growth vessel was assembled with the wick and circle of filter paper in place and the 25 ml volume of sand added. The basal salts solution was added to the top of the sand. The assembly was placed in a 250 ml beaker covered with aluminum foil and autoclaved. The dry heat sterilized mineral sample was spread on top of the sand and covered with 5 ml of separately sterilized sand. Seeds were planted by pushing below the surface of the sand to the level of the mineral sample. The assembly was transferred to the wide mouth jar certification chamber.

The following is a periodic progress report summarizing the most recent results of work done under contract NAS9-12050.

A number of standard procedures have been employed in the course of this project's work. In the present report these various procedures are presented together in detail for future reference.

Errors to be Avoided in Preparation of Tissue Homogenates.

If tissues are to be analyzed for total nitrogen, the homogenizing solution should not contain a N-containing buffer such as Tris. Use either bicarbonate or homogenize un-buffered in very clean water. Phosphate buffers should be used only if phosphorus and phosphatase are not to be analyzed.

Pipetting of tissues should be done while continuously agitating the homogenate. Solids tend to collect on sides of containers; therefore, sides should be washed down occasionally during the pipetting process. When using pre-set pipetts such as the Eppendorf, the orfice of the removable tip should be enlarged by trimming.

Whenever possible, pipeting of samples should be done immediately after the homogenization and not after the tissue has been frozen and thawed. Pipet a large number of samples of a standard size, 1 or 2 ml for the most enzyme assays or total protein determinations are easily thawed.

Sample containers present some problems. The polypropylene plastic #2006 tube supplied by Falcon Plastics is very useful. It will withstand centrifugation up to 10,000 rpm in the cold in the Sorval SS 34 head and can be used directly for all extractions described. It can be sealed

tightly with the molded plastic cap. It has the disadvantage that it is a good insulator and requires considerably more time and effort for thawing stored tissue as well as longer to freeze initially. Particularly, when working with plastic tubes all work should be done in an ice bath rather than in cracked ice.

Glass containers such as small serum vials are better than larger tubes because of the problem of encountered with tissue collecting on slides. Smaller culture tubes are also too small to allow the entrance of Eppendorf pipette tips.

Preparation of Cultured Tissue Homogenates.

The homogenizing medium has been either glass distilled water or Tris-maleate buffer pH 6.0 prepared as follows:

Tris-maleate - 24.2 g of Tris + 23.2 maleic acid made to 1 liter -- proportions - 50 ml Tris-maleate + 26 ml of 0.2 N NaOH (8 g/liter) made to 200 ml, $MgSO_4$ $7H_2O$ -- .739 g/liter

Sodium Ascorbate -- 1 mg/ml, i.e., 1g/liter

Tissue, 2.500 g + 10 mg is weighed into a Potter-type glass homogenizing vessel and 3.5 ml of ice cold homogenizing medium is added. The vessel is hereafter kept immersed in an ice bath. Homogenization is for 3 minutes at approximately 600 rpm with the teflon pestle. This results in a very thick homogenate which must stand several minutes before pipetting to allow bubbles to rise to the top. Homogenates are frozen over night before being used for enzyme assays.

Procedure for Extraction of Tissue in Preparation for Lowry Assay of Total Protein.

1. Pipet 0.5 ml of tissue homogenate into duplicate Corex centrifuge tubes, 15 ml round bottom acid cleaned (Alconox messes up Lowry reaction).

2. Pipet into each tube 5 ml of cold methanol; eject forcibly to accomplish mixing.
3. Allow to settle and extract in the cold overnight.
4. Centrifuge 5000 rpm for 5-10 min. and remove the supernatant with a long tipped pipet (Warburg pipet).
5. Add 5 ml more of methanol at room temperature. Mix pellet and centrifuge as before. Repeat this procedure twice more.
6. Dry off the remaining methanol with a stream of clean air or nitrogen. Do not dry completely; retain a moist pellet.
7. Add to the pellet 0.5 ml of 1.0 NaOH. Stir carefully, and allow to stand overnight; then mix vigorously on a vortex mixer.
8. Add 4.5 ml of distilled water and stir again on the vortex mixer.
9. Pipet samples for Lowry assay from the supernatant; generally about 100 ul will do for the materials we are working with.

Standard Curves.

Routinely all standards were prepared in duplicate at varying concentrations. The absorbance readings and corresponding known concentrations were then substituted in the simple regression formula. This procedure afforded increased precision over the use of a single set of standards and eliminated some of the tedium of graphically plotting and reading a standard curve. The method of calculation is given below in a cookbook form for the technician:

x = Concentration of standard

y = Absorbance reading for the colorimeter corresponding to that concentration

Calculate

Σx = Total of all x's $(\Sigma x)^2$ = This value squared.

Σx^2 = Square each x and add up all x^2's.

$\bar{x}$ = Average of all x's.

Σy^2 = Square each y and add up all y^2's.

$\bar{y}$ = Average of all y's.

Σxy = multiply each x by its corresponding y and add all to get a total.

n = Total number of values

$$m = \frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{n\Sigma x - (\Sigma x)}$$

$$b = \bar{y} - m\bar{x}$$

To find the amount of unknown corresponding to an observed absorbance reading substitute into the formula $x = \frac{y-b}{m}$.

Procedure for Total Protein by the Lowry Method.

The procedure given is the one that worked in our hands after modification to meet the requirements of this project. It is taken from: Lowry, et al. 1971, J. Biol. Chem. 193:265.

Reagents:

A.. 2% NA_2CO_3 in 0.1 N NaOH

B. 0.5% $CuSO_4$ 5 H_2O in 1% NaK-tartrate. Make to pH 7.2 to eliminate precipitate

C. Mix 50 ml of A and 1 ml of B. This solution should be discarded after 1 day

D. Stock Folin-Ciocalteau reagent from Fisher Chemicals Dilute 1:1 with distilled water just prior to use.

Standard solution: 300 ug Bovine Serum Albumin (BSA) Sigma, in 0.1 N NaOH

Procedure:

1. Pipet into an 18 x 150 mm glass tube a suitable volume of the extracted NaOH solution of protein from the extraction procedure given, and make to 1 ml with 0.1 NaOH
2. Add 5 ml of the copper carbonate solution C; mix and allow to stand for 10 min.
3. After 10 min. add and mix 0.5 ml of the 1:1 diluted Folin-Ciocalteau reagent from E
4. Allow to stand 30 min. then read absorbance at 750 nm
5. Compare with standards prepared by diluting 0.1 to 0.6 ml of the BSA standard to 1 ml and reacting as given above for the unknowns
6. Calculate protein (as BSA equivalents) by:
   a. Inserting the standard values into the regression formula given: or
   b. Picking a value on the curve. We used the OD for 100 ug of BSA which is usually .37 to .40 after blank subtraction Calculate as: $\frac{\text{OD standard}}{\text{OD unknown}}$ = x 100 ug BSA x the various dilution factors

Procedure for Soluble Phenols Using the Folin-Ciocalteau Reagent.

Soluble phenolics are removed in the methanol extracts of the procedure given above. They are then estimated by a procedure similar to the one employed in total protein determination.

Reagents:

A. Saturated NA_2 CO_3. Prepared by disolving 50 g of NA_2CO_3/ 100 ml water and heating to 75 C. Cool to room temperature for several hours; then seed with a few crystals of Na_2CO_3 10 H_2O. Filter and store in a brown bottle

B. Stock Folin-Ciocalteau reagent from Fisher Chemicals. Dilute 1:1

with distilled water just prior to use.

Standard solution: Resorcinol prepared to 10 ug/ml

Procedure:

1. Pipet into an 18 x 150 mm glass test tube a suitable volume (0.1 ml to no more than 0.5 ml) of the methanol extract and make to 4.5 ml with water.
2. Add 0.25 ml of the phenol reagent B and mix. Allow to stand for 2 minutes
3. Add 0.5 ml of saturated Na_2CO_3 reagent A and mix. Allow to stand for 60 minutes. Read absorbance to 725 mm.
4. Compare to a standard curve prepared by pipetting 0.1 to 1.0 ml of the standard solution of resorcinol, a volume of methanol equal to the amount used for the unknowns, and water to make 4.5 ml. Develop color in these along with the unknowns
5. Calculate total soluble phenols (as rescorcinol equivalents) by: a) inserting values from the standard curve into the regression formula given above, or b) picking on absorbance value near the middle of the range, approximately OD = .30, and calculating as: $\frac{\text{OD standard}}{\text{OD unknown}}$ x Concentration of standard x the various dilution factors.

Estimation of Total Phosphorus.

Total phosphorus is estimated by the Fiske-Subbarow colorimetric procedure after digestion in concentrated H_2SO_4.

Reagents:

10 N H_2SO_4 - add 300 ml of concentrated H_2SO_4 to a volumetric flask and make up to 1 liter with distilled water.

B. Hydrogen peroxide-30%. Test for phosphorus content.

C. Ammonium Molybdate - 2.5% in distilled water.

D. Elon-Bisulfite - 10 g Elon (p-methylaminophenol sulfate) Eastman P619 and 30 g of sodium bisulfite in 500 ml of distilled water. The solution used in our work remained stable for several weeks. The batch of Elon was better than usual.

Standard solution: K_2HPO_4 standard - oven dired salt (120 C, 2 hr) was weighed to give 1.247 g. This was made to 1 liter with distilled water. The solution was then diluted 1:10. A 0.5 ml volume of the second solution gave 10 ug of phosphorus. By the awway used in these analyses, this amount yield an average absorbance of 0.28.

Procedure:

1. Pipet triplicate samples (100 ul) into pyrex 15 x 85 mm tubes. At this same time pipet water blanks and 0.5 ml (10 ug of phosphate) of standard and dijest in parallel with the unknowns.
2. Add 0.8 ml of 10 N H_2SO_4
3. Place samples in a cold oven and turn heat to 150 C for 2 hr
4. After water has evaporated, turn over to 200 C. Periodically remove tubes from the oven and add 30% H_2O_2 to cooled tubes. Continue alternate heating and H_2O_2 addition until the digests are clear or slightly yellow. Heat at least 30 min. after the last H_2O_2 addition to ensure removal of all H_2O_2 which interferes with the color reaction.
5. Remove tubes from oven, cool and add 2 ml of distilled water. Heat in a boiling water bath for 20 min. to hydrolyze the pyrophosphates.
6. Add 2 ml of Ammonium molydate and 1 ml of Elon-bisulfite and

mix by inverting. Cover to invert with a piece of Parafilm. Add reagents to consecutive tubes at 30 sec. intervals.

7. Incubate for 15 min at 37 C. Withdraw tubes from the bath at 30 sec intervals in the same order that they were added
8. Read absorbance at 650 nm
9. Calculate phosphorus concentration as:

$$\frac{\text{absorbance of standard}}{\text{absorbance of unknowns}} \times 10 \text{ ug} \times \text{appropriate dilution factors}$$

Phosphatase Analysis

Introduction:

In this phosphatase assay, p-nitrophenol esters of phosphate are used as the enzyme substrate. The esters are colorless but the p-nitrophenol released by the cleavage of the phosphate is colored under alkaline conditions and serves as a direct index of phosphorus release. This results in considerable time savings over assays in which the phosphorus is measured. The procedure, based upon that given in the Sigma 104 bulletin, gives results equal or superior to those obtained by B-glycerol Phosphate as a substrate.

Materials:

A. Chemicals and Solutions

1. Buffer - use 0.5 ml/assay

a. Acid
10 ml 1 M Acetate buffer pH 5.0
20 ml 1% Triton X-100 v/v
25 ml 1M Sucrose
10 ml .03M $MgSO_4$
35 ml Distilled water

b. Alkaline
10 ml 1M tris HCl pH 8.5
20 ml 1% Triton X-100 v/v
25 ml 1M Sucrose
10 ml 0.03M $MgSO_4$
35 ml Distilled water

2. Substrated Solutions - 0.5 ml/assay

a. p-nitrophenyl phosphate (Sigma 104)
100 mg/25 ml water
b. bis-p-nitrophenyl phosphate Na salt (Sigma N-3002)
100 mg/25 ml water

NOTE: Sigma 104 is a phosphatase substrate, whereas, Sigma N-3002 is a diesterase or cyclic phosphatase substrate.

3. Substrate Solutions - 0.5 ml/assay

   a. 0.01 M p-nitrophenol (Sigma 104 - 8 Spectro-grade) store in refrigerator
   b. NaOH solution
      0.1N - 4g NaOH/liter
      0.02N - dilute 0.1N NaOH 1:5 with distilled water

4. Buffers for pH Curves (Table 1)

B. Apparatus

1. Eppendorf Pipets

   a. #22-34-280-10 500 ul for dispensing substrates and buffers
   b. #22-34-160-0 100 ul for dispensing enzyme prep or homogenate

2. Labindustries Repipets

   a. #3001 1 ml capacity for dispensing substrates and buffers as an alternate to 500 ul Eppendorf
   b. #3010A 10 ml capacity for delivering base to kill reaction and develop color (always wash out after use)
   c. #81001 Automatic dilutor with 1 ml aspirator syringe and 10 ml dilutor syringe for diluting samples for reading color

3. Test tubes

   a. Corex 15 ml round bottom centrifuge tubes or Falcon #2006
   b. Falcon #2006 polypropylene tubes

4. Water Bath - set at 37 C

5. Stopwatch

Methods.

A. Incubation conditions

1. Temperature: 37 C

2. Time: 20 minutes

3. Replications - duplicate or triplicate tubes + one substrate blank. (Enzyme blanks are not necessary and substrate blanks will be near zero provided the tubes are put on ice immediately after additon of the NaOH).

4. pH: 5.0 and 8.0

5. pH Curves: reactions are carried out in the same manner as those at pH 5 and pH 8. Buffers are listed in Table 1.

B. Procedures

1. Thaw the homogenate with continous shaking and hold ice. (This is probably an unnecessary precaution for acid phosphatase because activity remains high over several days of incubation at 37 C).

2. Pipet into tubes on ice 0.5 ml of the buffer and 0.1 ml of enzyme. (Keep no longer than 15 minutes before addition of substrate. During pipetting scrape solid materials from the sides of the tube and stir constantly. See also alternative procedure)

3. On a timed schedule add substrate (0.5 ml) to tubes at 10 to 20 second intervals (whatever is convenient). Tubes are equilibrated at the incubation temperature for 1 minute prior to substrate addition. For example:

   Time
   -60 sec-----place tube 1 in water bath
   -40 sec-----place tube 2 in water bath
   -20 sec-----place tube 3 in water bath
   0---------add substrate to 1 and place tube 4 in water bath
   +20 sec-----add substrate to 2 place tube 5 in water bath
   +40 sec-----add substrate to 3 and place tube 6 in water bath
   +60 sec-----etc.--continue in this manner until substrate is added to all tubes

4. After 20 minutes incubation begin adding 5 ml 0.1 N NaOH to kill reaction and develop the color using the same time schedule as was used for addition of substrate. Place tubes on ice as soon as base has been added to prevent high blanks

5. Using an automatic dilutor, dilute (usually 1:5 or 1:10) the reaction mixtures as needed, if necessary, and read color at 410 nm

6. Prepare a standard curve from 0.01 M solution of p-nitrophenol added to 0.02 M NaOH

C. Preparation of Standard Curve

1. Dilute 0.5 ml of p-nitrophenol standard solution (0.01M) to 100 ml in 0.02N NaOH

2. Add the following amounts to a series of tubes:

Tube	ml	uM	uM/ml	Blank Subtracted OD
1	0.5	.025	.0025	.036
2	1	.050	.0050	.075
3	2	.100	.010	.163
4	3	.150	.015	.254
5	4	.200	.020	.343
6	5	.250	.025	.442
7	0	.000	.000	.000

make all tubes to 10 ml

D. Alternative Procedure

In cases such as the running of pH curves where the same homogenate is used for a large number of tubes, it is often more convenient to start the reaction by adding the enzyme. In this case substrate and buffer are pipetted together, equilibrated as described, and the homogenate added on a time schedule. The results from this procedure have not been found to differ significantly from the other.

E. Calculation of Activity

$$\frac{\text{Unknown - Bk}}{\text{Standard -Bk}} \times (\text{uM nitrophenol/ml of standard}) \times (\text{dilution factor})$$

$$\times 6.1 \times 10 \div 20 \div \text{mg N/ml homogenate, or mg protein/ml homogenate,}$$

or mg dry wt/ml homogenate = enzyme units/mg N or protein

or dry weight.

dilution factor - generally 1:5 or 1:10
6.1 = total amount of reaction mixture
10 = gives the amount of substrate released/ml of homogenate
20 = converts to a per minute basis

TABLE I

PREPARATION OF BUFFER SOLUTIONS FOR pH CURVE[a]

pH	Buffer Components[b] (ml)	
3.2	Citric Acid (0.1M)	Sodium Citrate (0.1M)
	43.7	6.3
4.2	31.5	18.5
	Acetic Acid (0.2M)	Sodium Acetate (0.2M)
4.0	41.0	9.0
4.8	20.0	30.0
5.6	4.8	45.2
	Acid Sodium Maleate (0.2M)	NaOH (0.2M)
6.0	25.0	13.4
6.6	25.0	20.8
	Tris-Maleate (0.2M)	NaOH (0.2M)
6.4	25.0	18.5
7.0	25.0	24.0
7.6	25.0	29.0
8.0	25.0	34.5
	Tris (0.2M)	HCl (0.2M)
7.4	25.0	20.7
8.2	25.0	10.9
9.0	25.0	2.5
	Glycine (0.2M)	NaOH (0.2M)
9.0	25.0	4.4
9.6	25.0	11.2
10.4	25.0	19.3

a

Preparation of buffer components

0.1 M Citric Acid----------21.01 g/l
0.1 M Sodium Citrate---------29.41 g/l of $C_6H_5O_7Na \cdot 2H_2O$
0.2 M Acetic Acid----------11.55 ml/l
0.2M Sodium Acetate----------16.4 g/l of anhydrous or 27.2 g/l trihydrate
0.2M Acid Sodium Maleate-----8 g NaOH + 23.2 Maleic Acid/l
0.2M NaOH----------8 g/l
0.2M Tris-Maleate----------47.4 g/l or 24.2 g Tris + 23.2 g Maleic Acid/l
0.2M Tris----------24.2 g/l
0.2M HCl----------17.2 ml concentrated/l or 86 ml/l diluted 1:5
0.2M Glycine----------15.01 g/l

b

Prepare buffer solutions of desired pH by adding components in the amount given, 20 ml 1% Triton X-100, 25 ml 1.0M sucrose, and distilled water to make a final volume of 100 ml.

Harvesting and Preparation of Plant Tissue for Activation Analysis.

Because of the high sensitivity of activation analysis, considerable care must be taken in collection and preparation of samples. A major possible source of contamination is sodium from the fingers. Another is, in the case of the present work, from the mineral substrate being tested. A third is from the metal instruments employed in handling the plant material. Given below are the procedures and precautions employed.

Instruments. Forceps and scalpels employed in harvesting test plants were stainless steel. The scalpel found most practical was a stainless injector razor blade held in an Exacto handle. Both instruments were wiped with a clean Kimwipe after each use.

Containers. Weighing pans for drying samples were polystyrene (s/p, dispo) 3" x 3" used directly from the package without washing. (These were handled only by the rims to prevent sodium contamination of areas contacting the drying plant material. Storage of samples was in snap cap 2 dram polyethylene vials. These were rinsed in double distilled water and dried prior to use.

Harvest procedure. Individual fresh weight yields were measured for each growth vessel. Only a combined dry weight of each treatment was measured in order to reduce handling and the possibility of contamination. Plants harvested from gauze chambers, where there was greater chance of contamination from the substrate, were in some experiments washed by dipping in three successive rinses of double distilled water. No individual fresh weights were taken in these cases. With these exceptions the following outlines the procedure of harvesting all experimental plants:

1. Dry weighing pans at 75 C for at least 2 hours prior to weighing

2. Remove the weighing pan from the drying oven with forceps and weigh to the nearest milligram within 10 seconds of removal from the oven. Leave the pan on the balance and begin harvesting the plants of a given treatment.

3. Grasp the plant or plants gently with stainless forceps, lift gently and sever the stem just about the root collar or above the last point of contact with the substrate. Remove any leaves that have drooped and contacted the substrate.

4. Place the plants on the weighing pan, record the weight and number of plants and continue as rapidly as possible to harvest and weigh the other plants in the treatment, recording the cumulative total fresh weight as each new set of plants is added. Delay results in significant weight loss of plants already on the pan. Wipe the instruments with a clean Kimwipe between weighings.

5. Place the pan and plants in the drying oven and dry overnight (12-15 hr) to a constant weight. During approximately the first 2 hr of drying, use clean stainless forceps to bunch the plant tissue into an elongated compact mass that will fit inside the 2 dram polyethylene storage vials. Loose dried tissue is nearly impossible to handle without some loss.

6. Reweigh the tissue after drying as in 5. Record weights, remove tissue to storage vials, number and record oven dry weight along with the treatment and sample number.

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
LYNDON B. JOHNSON SPACE CENTER
HOUSTON, TEXAS 77058

REPLY TO
ATTN OF: DD52-L148-73

JUN 13 1973

R. S. Halliwell, Ph. D.
Department of Plant Sciences
Texas A & M University
Texas Agricultural Experiment Station
College Station, TX 77843

Dear Doctor Halliwell:

The literature search on mineral nutrition and plant tissue cultures that you furnished as part of your work on Contract NAS9-12050 have been received. The copies of key papers which you have supplied have been useful. In view of the time you have devoted to this effort, you should include a summary of the library research in bibliographic format in the final contract report.

Sincerely,

Charles H. Walkinshaw

Charles H. Walkinshaw, Ph. D.
Principal Plant Pathologist
Health Services Division

cc:
BB321/P. R. Kimbrough

"SELECTED REFERENCES OF INTEREST IN RELATION TO PREPARATION OF LUNAR SOIL FOR AGRICULTURAL PURPOSES"

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